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**REVIEW ARTICLE**

# Type Six Secretion System (T6SS) in Aquatic Pathogens

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ABSTRACT

For aquaculture to overcome challenges in diseases, preventive and therapeutic interventions are needed. One solution of great potential is the use of bacterial nanomachines. Protein secretion systems facilitate nutrient acquisition, communication, and disease by delivering virulence factors. The Type VI secretion system (T6SS) is one of the protein secretion systems that is extremely widespread and targets bacterial and eukaryotic cells for fitness and pathogenicity. The T6SS function can be redirected as a target for vaccine development and therapeutics for aquaculture applications. Choosing a strain that encodes T6SS and understanding its function and activity is vital to accomplishing this. This review outlines the current knowledge on the function, organization, and regulation of T6SS in aquatic pathogens, including important fish and crustacean pathogens *Vibrio*, *Aeromonas*, *Edwardsiella*, *Flavobacterium*, *Pseudomonas,* and *Francisella*. Overall, the T6SS in *Vibrio* and *Edwardsiella* are the two well-studied groups to date. The review identifies research gaps and directs future studies to develop technologies to control diseases caused by pathogens of aquaculturally essential species. Future research and development on T6SS can be applied to important, newly emerging, and re-emerging bacterial, viral, and fungal diseases.

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## 1. INTRODUCTION

Many bacterial pathogens use various protein secretion systems to deliver virulence effectors into hosts. Type III, IV, and VI secretion systems are important virulence determinants (Yu and Lai 2017). The Type VI Secretion System (T6SS) is encoded by about 25 % of Gram-negative bacteria, including human, animal, and plant pathogens and non-pathogens (Bingle et al. 2008).

In all T6SS identified to date, 13 conserved and essential genes comprise a core gene cluster common among all species (Boyer et al. 2009). The core components of the machinery are composed of membrane complex (*tssJ*, *tssL*, *tssM*), baseplate (*tssK*, *tssE*, *tssF*, *tssG, vgrG*), tube (*hcp*), and contractile sheath (*tssB*, *tssC*) that surround the tube and attaches to the baseplate (Brackmann et al. 2017). ClpV is a disassembly ATPase that provides energy for the machine.

The assembly starts with membrane complex formation to position the baseplate. The baseplate serves as a platform for contractile tail elongation. The tube is built by stacked Hcp rings and tipped with a puncturing VgrG and PAAR encoding protein (Shneider et al. 2013). Sheath contraction propels the VgrG, carrying the effectors toward the target. After delivering effectors, the sheath is disassembled, and subunits are recycled to build another one (Figure 1). In most aquatic pathogens encoding T6SS, the T6SS gene cluster is designated as *tss* or type six secretion (*tssA-tssM*). In *Edwardsiella*, the T6SS is called the Edwardsiella virulence protein (EVP) gene cluster encoding *evpA-evpO*. While in *Vibrio anguillarum* and *V. crassostreae*, this is called Vibrio type six secretion encoding *vtsA*-I. The homologs in T6SS gene clusters discussed in this review are summarized in Table 1.

T6SS confers a competitive advantage against bacterial competitors, helps in adapting to stress conditions (Yu et al. 2021), and facilitates metal ion uptake (Lin et al. 2017). Recent studies have shown

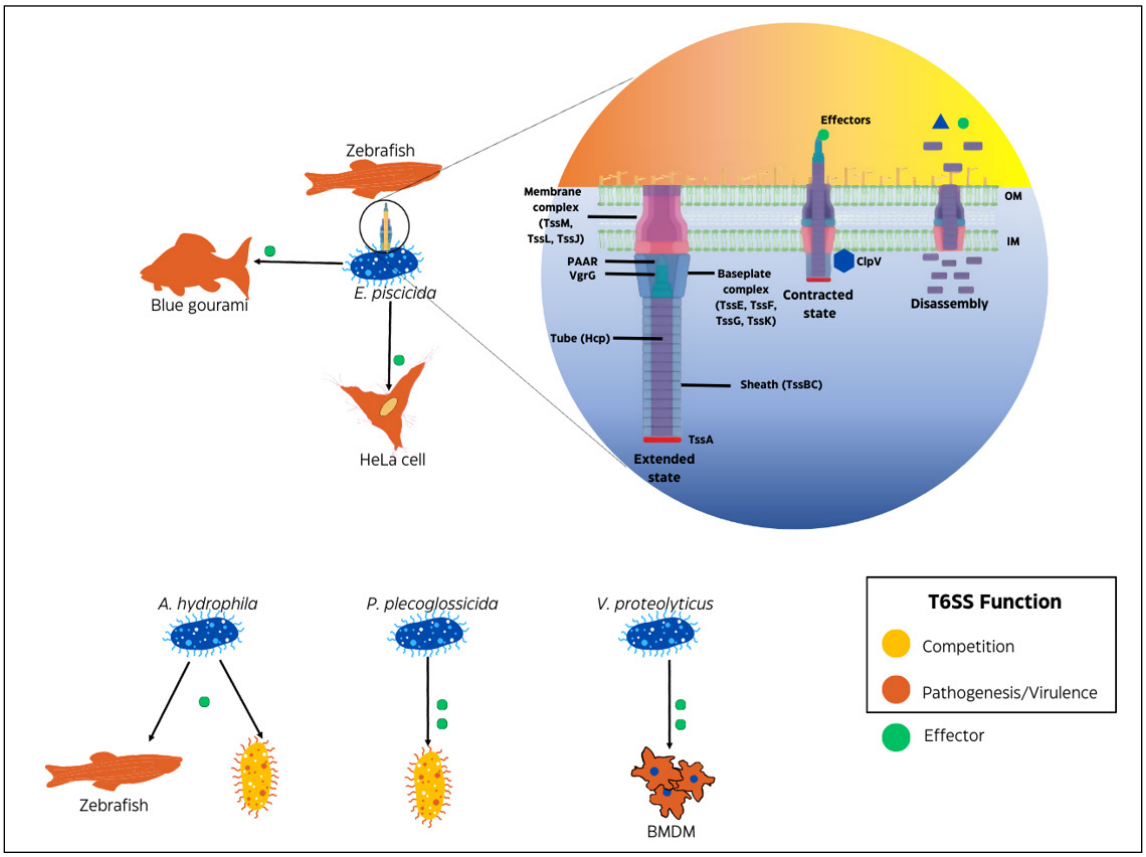


**Figure 1.** The Type VI Secretion System components, assembly and functions in aquatic pathogens. T6SS encoding aquatic pathogen (blue) delivers effectors (green circle) to other bacterium for inter-bacterial competition (yellow) and eukaryotic cells for pathogenesis (orange) as shown by *in vitro* (i.e. HeLa cells, bone marrow-derived macrophages or BMDM) and *in vivo* fish infection model discussed in this review.

a wide distribution of interbacterial T6SS in *Vibrio* species. In the colonization of squid, the *V. fischeri* T6SS is responsible for controlling competition (Speare et al. 2018). T6SS has also been reported to be involved in pathogenesis (Pukatzki et al. 2006; Wang et al. 2022). One protein that contains the forkhead-associated (FHA) domain, TagH, is involved in regulating the virulence of *V. cholerae* (Wang et al. 2022). The T6SS of coral and shellfish pathogen *Vibrio coralliilyticus* mediates mortality in brine shrimp Artemia nauplii and macrophages (Mass et al. 2024). Additionally, it has been observed that T6SS influences the microbiome makeup of polymicrobial communities (Tang et al. 2022).

To date, there are few studies on the characterization of T6SS in pathogens causing diseases in aquaculture (Table 2). This paper discusses an overview of the current understanding of T6SS encoded in significant aquatic pathogens.

In addition, future perspectives are provided on the possible application of T6SS. Since diseases result in the largest financial losses in aquaculture, new disease prevention, management, and treatment approaches are urgently needed. The T6SS can be a delivery platform to target different locations (Allsopp and Bernal 2023). Using T6SS over other protein secretion systems for delivery has several advantages. First, it does not require a receptor to deliver effectors to the cytoplasm of the target cell. Secondly, in contrast to T3SS, which can only deliver unfolded proteins, T6SS can deliver folded proteins, their native conformation. Thirdly, it is extremely widespread, encoded by one-fourth of Gram-negative bacterial genomes, and has a wide array of target cells, targeting both eukaryotic and prokaryotic cells.

## *2. Vibrio sp.*

*Vibrio* is capable of causing diseases in a variety of marine animals, including corals, shrimp, mollusks, and fish, both wild and farmed (Rubio et al. 2019).

**Table 1.** Homologs of T6SS genes and their function described in this review. The T6SS gene cluster is called *Edwardsiella* virulent protein (Evp) in *Edwardsiella* spp; type six secretion (Tss) in *Vibrio parahaemolyticus* and *V. proteolyticus*; and Vibrio type six secretion (Vts) in *Vibrio anguillarum* and *V. crassostreae*.

| Edwardsiella spp. | *V. parahaemolyticus, V. proteolyticus* | *V. anguillarum, V. crassostreae* | Function |
|---|---|---|---|
| EvpA | TssA | | assembly chaperone and tube/sheath cap |
| EvpB | TssB | TssB | contractile sheath |
| EvpC | Hcp | Hcp | tube |
| EvpD | N/A | | |
| EvpE | TssC | TssC | contractile sheath |
| EvpF | N/A | | |
| EvpG | TssE | | baseplate |
| EvpH | TssH | TssH/ClpV | Sheath disassembly |
| EvpI | VgrG | VgrG | Spike |
| EvpJ | N/A | | |
| EvpK | N/A | | |
| EvpL | TssJ | VtsF/TssJ | membrane complex |
| EvpM | TssK | VtsG/TssK | baseplate |
| EvpN | TssL | VtsH/ IcmH/DotU | membrane complex |
| EvpO | TssM | VtsI/TssM/IcmF | membrane complex |
| N/A | TssF | TssF | baseplate |
| N/A | TssG | TssG | baseplate |
| N/A | N/A | VtsE | |
| N/A | N/A | VtsA | not found in other characterized T6SS |
| N/A | N/A | VtsB | not found in other characterized T6SS |
| N/A | N/A | VtsC | not found in other characterized T6SS |
| N/A | N/A | VtsD | not found in other characterized T6SS |

### *2.1 Vibrio parahaemolyticus*

Initial studies revealed that *Vibrio parahaemolyticus* encodes two T6SS, namely T6SS1 and T6SS2, encoded on chromosomes 1 and 2, respectively (Yu et al. 2012). All *V. parahaemolyticus* strains encode the T6SS2 gene cluster but not the T6SS1 (Jana et al. 2022). A recent pan-genome analysis identified two additional clusters with minimal distribution – T6SS3 and T6SS4, which may have been acquired through horizontal gene transfer (Jana et al. 2022). The third T6SS gene cluster was named T6SS3 because of its similarity in genetic structure to *V. proteolyticus* T6SS3 described in Ray et al. (2017). T6SS3 gene cluster homologs are also found in other pathogenic *Vibrio* spp., including *V. proteolyticus*, *V. vulnificus*, *V. splendidus*, *V. crassostrea* and *V. anguillarum* (Cohen et al. 2022). The T6SS4 in some strains are encoded either in a plasmid or chromosome, such as in *V. parahaemolyticus* strain VP157 and strain 160807, respectively, which are both isolated from white leg shrimp (*Penaeus vannamei*).

Yu et al. (2012) found differences in the role of T6SS1 and T6SS2 in cell adhesion. T6SS2 mediates adherence to HeLa cells, whereas T6SS1 is implicated in cell adhesion as evaluated using Caco-2 and HeLa cells. As a result of deleting the entire T6SS gene cluster 1 (*t6ss1)*, there was a reduction in bacterial adhesion to the Caco-2 cells. While in HeLa cells, both Δ*t6ss1* and Δ*t6ss2* showed decreased adhesion to HeLa monolayers. The difference in results may be due to the surface characteristics of the cells, such as receptors that interact with T6SS.

Li et al. (2017) demonstrated that the T6SS1 significantly varied across *V. parahaemolyticus* strains and found two sites—referred to as Sites 1 and 2. In shrimp, *Vibrio parahaemolyticus* is a major cause of acute hepatopancreatic necrosis disease or AHPND (Lai et al. 2015). It was suggested that T6SS is associated with AHPND. A comparative genome sequence analysis demonstrated that the T6SS gene cluster distinguishes between strains that cause AHPND and those that do not (Li et al. 2017). In *V. parahaemolyticus*, which does not cause AHPND, the

**Table 2.** Function of Type VI Secretion System in aquatic pathogens

| Organism | Function | Reference |
|---|---|---|
| ***Vibrio* spp.** | | |
| *V. vulnificus* | Antibacterial activity | Hubert and Michell, 2020 |
| *V. tasmaniensis* | Phagocytosis dependent cytotoxicity | Rubio et al., 2019 |
| *V. crassostrea* | No reported characterized function yet, only genome analysis | Bruto et al., 2017; Rubio et al., 2019, Cohen et al., 2022 |
| *V. parahaemolyticus* | Bacterial adhesion, pathogenicity in shrimp; antibacterial activity | Yu et al., 2012; Salomon et al., 2013; Wang et al., 2022 |
| *V. alginolyticus* | Antibacterial activity; bacterial adhesion, motility and pathogenicity to zebrafish | Yang et al., 2018; Wu et al., 2023 |
| *V. anguillarum* | Stress response and quorum sensing | Weber et al., 2009 |
| *V. harveyi* | Not characterized yet, only genome analysis; T6SS component as a putative antigen and fish vaccine | Fu et al., 2021; Hu and Sun, 2011; Sun et al., 2019 |
| *V. proteolyticus* | Antibacterial activity, toxicity to HeLa cells, actin rearrangements in yeast and macrophage cells; pathogenicity to brine shrimp Artemia salina nauplii | Ray et al., 2017; Cohen et al., 2023; Salomon et al., 2014 |
| ***Aeromonas* spp.** | | |
| *A. hydrophila* | Antibacterial activity, bacterial adhesion, biofilm formation, virulence to zebrafish, channel catfish and grass carp | Wang et al., 2018; Ma et al., 2020; Tekedar et al., 2019 |
| *A. veronii* | Antibacterial activity, virulence regulation, biofilm formation, bacterial adhesion, motility and pathogenicity to zebrafish and crucian carp | Song et al., 2020; Wang et al., 2023 |
| *A. salmonicida* | Bacterial adhesion, biofilm formation, extracellular product secretion and pathogenicity to grouper | Cai et al., 2022 |
| ***Edwardsiella* spp.** | | |
| *E. piscicida* | Fish phagocyte regulation, virulence to blue gourami, zebrafish, Japanese flounder and blue gourami; and bacterial translocation/invasion/colonization, calcium flux | Rao et al., 2004; Zheng and Leung, 2007; Hu et al., 2014; Wang et al., 2009; Tan et al., 2019; Zhang et al., 2018; Li et al., 2021; Chen et al., 2017 |
| *E. ictaluri* | Pathogenicity to catfish fingerlings, bacterial adhesion, colonization, internalization, replication | Rao et al., 2004; Abdelhamed et al., 2018; Kalindamar et al., 2020; Kalindamar et al., 2021; Kalindamar et al., 2023 |
| *E. anguillarum* | Not characterized yet, only genome analysis | Shao et al., 2015 |
| ***Pseudomonas* spp.** | | |
| *P. plecoglossicida* | Pathogenicity to large yellow croaker, grouper, biofilm formation, motility, bacterial adhesion and replication, antibacterial activity, chemotaxis, | Tao et al., 2020; Huang et al., 2019; Li et al., 2022; Luo et al, 2019; Yang et al., 2023; Wang et al., 2018; Jin et al., 2021 |
| ***Francisella* spp.** | | |
| *F. noatunensis* subsp. orientalis | Pathogenicity to zebrafish | Hansen et al., 2021 |
| ***Flavobacterium* spp.** | Not characterized yet, only genome analysis | Tekedar et al., 2017; Kumru et al., 2020 |

T6SS gene is not present.

In another study, Yang et al. (2019) examined genetic variations among 15 strains of *V. parahaemolyticus* and found that the genetic structure of T6SS1 is genotype-dependent. Wang et al. (2022) demonstrated that T6SS gene clusters in *V. parahaemolyticus* may be horizontally acquired between chromosomes and plasmids. The comparison of genes from chromosomes and plasmids determined that no distinct groups of T6SS core genes exist..

The T6SS1 of *V. parahaemolyticus* has been characterized as an antibacterial system in pathogenic isolates, predominantly in AHPND-causing isolates. It has been demonstrated that T6SS1 is active under marine-like conditions, whereas T6SS2 is active under low salt conditions, as demonstrated by the production of Hcp1 and Hcp2 under such conditions (Salomon et al. 2013). Further, the T6SS-mediated killing is strain-specific, targeting V. cholerae, E. coli, Yersinia pseudotuberculosis, and *Vibrio*

*natriegens* but not *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens*. The T6SS gene cluster of *V. parahaemolyticus* VP157, isolated from white-leg shrimp (*Penaeus vannamei*), is encoded in a plasmid, and the antibacterial activity is T6SS-dependent (Wang et al. 2022). In a co-culture assay, the wildtype exhibited higher antibacterial activity against gram-negative bacteria *(V. cholerae and E. coli)* and gram-positive bacterium *(Bacillus pumilus),* compared to the T6SS deletion mutant strain (Δ*t6ss*). A mock microbial community comprising seven commonly found bacteria in shrimp ponds was used to test the survival of wildtype and T6SS deficient strains. Results showed that the growth wildtype was significantly higher, and it is T6SS dependent, suggesting that T6SS may be able to alter the microbiome compositions of shrimp ponds (Wang et al. 2023a).

For the regulatory mechanisms of *V. parahaemolyticus*, each T6SS cluster is differentially regulated by quorum sensing. T6SS1 and T6SS2 are negatively and positively regulated by the quorum sensing master regulator OpaR (Salomon et al. 2013).

### 2.2 *Vibrio vulnificus*

Three biotypes are recognized based on the biochemical traits of *Vibrio vulnificus*. Biotype 2 causes warm-water vibriosis, an acute hemorrhagic septicemia affecting eels and other teleost. Several important aquaculture species are susceptible to *V. vulnificus*, including eels (*Anguilla* sp.), tilapia (*Oreochromis* sp.), grass carp (*Ctenopharyngodon idella*), trout (*Oncorhynchus mykiss*), pompano (*Trachinotus ovatus*) and shrimp (*P. vannamei*) (Amaro et al. 2015; Janampa-Sarmiento et al. 2024). The genome of *V. vulnificus* encodes T6SS1 and T6SS2, each with 13 core genes (Church et al. 2016). Interestingly, only a small number of *V. vulnificus* strains had T6SS1. However, all sequenced strains have been shown to have T6SS2.

Functional characterization revealed that *V. vulnificus* T6SS plays an important role in interspecies and intraspecies competition in oyster hosts (Hubert and Michell 2020). The antibacterial activity is thermoregulated; T6SS1 and T6SS2 are active at 21$^0$C, but only T6SS1 is active at 30$^0$C. When the wildtype *V. vulnificus* 106-2A was used to compete at 30$^0$C, it reduced the survival of both *V. vulnificus* 99-743 and *Salmonella enterica serovar* Enteritidis CC012 by 1000-fold. There is a T6SS dependency, as no reduction was observed with the T6SS deletion mutant (Δ*hcp1*). On the other hand, a 10-fold reduction in the survival of target *V. vulnificus* 99-743 was observed when cocultured with wildtype strain and no reduction with T6SS2 deletion mutant (Δ*hcp2*; Δ*icmF2*) at 21$^0$C (Hubert and Michell 2020; Church et al. 2016). The IcmF (intracellular multiplication protein F), initially identified as a T4SS component, shares similarities with TssM (Bonemann et al. 2010; Li et al. 2019). Further, no killing was observed when cocultured with the deletion of T6SS1 and T6SS2 (Δ*hcp1*Δ*hcp2*). Interestingly, the reduction of the killing of *S. enterica serovar* Enteritidis CC012 can only be observed in Δ*hcp1*Δ*hcp2,* and no significant difference can be observed between the wildtype, Δ*hcp1* and Δ*hcp2* alone. Aside from thermoregulation, T6SS in *V. vulnificus* is negatively regulated by increasing salinity concentrations (Church et al. 2016).

There is no T6SS-dependent anti-eukaryotic activity in *V. vulnificus* when tested against *Dictyostelium discoideum* and *Galleria mellonella* (Hubert and Michell 2020; Church et al. 2016). Using plaque assay, plaques were observed on T6SS deletion mutants Δ*hcp1* and Δ*hcp2*, indicating predation by *D. discoideum* (Hubert and Michell 2020). The virulence is not attenuated in Δ*icmF1* and Δ*icmF2* in the *G. mellonella* infection assay (Church et al. 2016).

### *2.3 Vibrio tasmaniensis*

After being isolated from diseased oysters, the virulence of *V. tasmaniensis* was confirmed in oyster infection experiments (Gay et al. 2004). The hemocyte intracellular pathogen *V. tasmaniensis* LGP32 encodes the T6SS1 and T6SS2 gene clusters. T6SS1 is encoded in three other virulent *V. tasmaniensis* strains analyzed and absent from avirulent strains (Rubio et al. 2019). All the virulent strains contain an ortholog *evpP*, a T6SS effector in *Edwardsiella tarda*. T6SS is induced upon oyster colonization. As a result of inactivating *vipA* in the T6SS1 gene cluster, oyster experimental infections were rendered non-virulent, and no cytotoxicity on oyster hemocytes was observed. VipA is a homolog of TssB, a component of the T6SS contractile sheath. This implies that the cytotoxicity depends on phagocytosis and necessitates the T6SS. In contrast, T6SS2 inactivation showed no effect on cytotoxicity or virulence, indicating that it has no effect on the host-pathogen interaction.

### *2.4 Vibrio crassostreae*

The T6SS genes are present in all *V. crassostreae* virulent strains, including J2-9, J5-4, LGP8, and J5-20 (Rubio et al., 2019). In the virulent

strain J2-9, T6SS is found on pGV1512, the virulence plasmid for *V. crassostreae*. The T6SS gene cluster consists of *vtsA-vtsI*. The T6SS was induced eight hours after the experimental infection of the oyster (*Crassostrea gigas*) with J2-9. Further, the mobile genetic elements involved in conjugative transfer were also highly induced. This corroborates with Cohen et al. (2022), wherein the T6SS gene cluster is encoded next to mobile elements, including integrase or transposase.

A T6SS deletion was generated in *V. crassostreae* strain J2-9 using allelic exchange and assessed for virulence to specific pathogen-free (SPF) oysters. The T6SS deletion did not affect the virulence of *V. crassostreae* after 24 hours post-infection (Bruto et al. 2017).

### *2.5 Vibrio alginolyticus*

The T6SS expression in *V. alginolyticus* is mediated by quorum sensing (QS) regulators and alternative sigma factors (Sheng et al. 2012). LuxO provides positive and negative regulation, which is provided by LuxR and LuxS. Moreover, the enhancer-binding protein VasH and the alternative sigma factor RpoN control the expression of T6SS1. A mutation in RpoN or VasH caused a decrease in the expression of Hcp1, suggesting that those genes are positive regulators of Hcp1. In addition, LuxR, RpoN, and VasH may play a role in regulating the expression of other T6SS genes, such as *clpV1*, *icmF,* and *vasA1,* a homolog of *tssF* (Sheng et al. 2012).

T6SS1 and T6SS2 are present in the strain EPGS of *Vibrio alginolyticus* (Sheng et al. 2013). The T6SS2 gene cluster encodes the phosphatase PppA that participates in a complex regulatory network that also involves the T6SS substrate Hcp1, quorum sensing, and 30,50-cyclic diguanylic acid (c-di-GMP) (Sheng et al. 2013). Whole genome transcriptome analysis has revealed a number of PppA regulatory targets, including flagellar proteins, exotoxin alkaline serine protease (Asp), quorum sensing regulator LuxR, Hcp, and proteins involved in polysaccharide biosynthesis and transport. PppA negatively regulates the Hcp1 expression and secretion. The deletion of *pppA* could induce the transcription and protein expression of *hcp1*. Upon deletion of *pppA*, c-di-GMP expression increased, and LuxR and the exotoxin Asp decreased. Further, cell aggregation, increased biofilm formation, and reduced swarming were observed in Δ*pppA*.

The fish-isolated *V. alginolyticus* EPGS has T6SS2-antibacterial activity against *E. coli*, *V. alginolyticus*, and *Edwardsiella* (Yang et al. 2018). The bacterial competition assay against *E. coli* showed that Δ*hcp1* had a similar killing as wildtype strain. The Δ*hcp2* and Δ*hcp1*Δ*hcp2* were unable to kill, but killing was restored when *hcp2* was complemented in Δ*hcp1*Δ*hcp2* strain. PpkA2, a serine-threonine kinase PpkA homolog encoded in *V. alginolyticus* T6SS2, modulates antibacterial activity and Hcp2 secretion. The Δ*ppkA2* was defective in killing *E. coli*. Moreover, the activation of T6SS2 is controlled by PpkA2 phosphorylation and quorum sensing. The phosphoproteomic analysis showed the substrates of the T6SS regulator PpkA2 kinase. T6SS2 function depended on PpkA2 and VtsR (*Vibrio* type six secretion regulator) phosphorylation. It is believed that VtsR phosphorylation controls the expression of the quorum sensing (QS) gene by regulating the expression of LuxR, one of the key regulators of QS. This suggest that PpkA2 phosphorylation cascade regulates T6SS and quorum sensing pathways in concert since LuxR is required for the expression of T6SS2 (Yang et al. 2018).

Wu et al. (2023) showed that the T6SS of *V. alginolyticus* is involved in mediating motility, adhesion, and pathogenicity by impacting its flagellar system. The *V. alginolyticus* HY9901 with Δ*hcp2* deletion impaired the swarming motility, reduced adhesion, and attenuated the virulence against zebrafish. Transmission electron microscopy (TEM) revealed abnormal morphology of flagella, the Δ*hcp2* had severely hollow-like structure of flagellar filament. Further, the levels of three flagellum FlaA, FlaB, and FlaC protein and assembly-associated proteins (i.e. FliH, FliF and FlgE) were decreased in Δ*hcp2*. The transcription of the three flagellum genes and sigma factors *rpoN*, *fliA*, and *rpoS* were also reduced in the Δ*hcp2* strain.

### *2.6 Vibrio anguillarum*

*Vibrio anguillarum* affects freshwater, brackishwater and marine fish, crustaceans, and bivalves, resulting in losses to the aquaculture industry (Frans et al. 2011). Chemotactic motility and adhesion, hemolysin, metalloproteases, iron absorption system, lipopolysaccharides, outer membrane proteins, quorum sensing, and sigma factors are among the virulence factors that have been found (Guanhua et al. 2018).

Based on the whole genome sequencing, it has been reported that *V. anguillarum* MVM425, a highly pathogenic strain isolated from moribund turbots in China, encodes T6SS (Guanhua et al. 2018).

The *V. anguillarum* T6SS modulates stress response and quorum sensing (Weber et al. 2009). *V. anguillarum* strain NB10 serotype O1, isolated from a diseased fish, contains VtsA-H (vibrio type six secretion) proteins. The proteins VtsA-D are not found in other characterized T6SS. The sequence and protein secretion analysis of VtsE-H revealed that these are T6SS components. The VtsE-H are homologs of the forkhead-associated protein, outer membrane lipoprotein TssJ, TssK, IcmH-related protein/ DotU, and TssM, respectively. DotU is a conserved inner membrane component of T6SS, a T4SS-like component similar to ImpK/TssL (Filloux et al. 2008). T6SS regulated the expression of metalloprotease EmpA and PrtV. The protease activity decreased in Δ*vtsB*, Δ*vtsE-H,* and Δ*hcp* and increased in Δ*vtsA*, Δ*vtsC,* and Δ*vtsD*. This regulation occurs as T6SS positively regulates the expression of RpoS, a stress response regulator, and VanT, a quorum sensing master regulator, which then regulates the protease expression. A study of T6SS mutants exposed to hydrogen peroxide, ethanol, or low pH was conducted to explore pigment production, *hpd* expression, and survival after exposure to these conditions. The gene *hpd* encodes a 4-hydroxyphenylpyruvate dioxygenase and functions in pigment production. T6SS may control the stress response since stress indicators increased in Δ*vtsA*, Δ*vtsC*, and Δ*vtsD* and decreased in Δ*vtsB*, Δ*vtsE–H*, and Δ*hcp*. In addition, no attenuation of virulence was observed when rainbow trout were infected with *vtsA-H* mutants.

### *2.7 Vibrio harveyi*

*Vibrio harveyi* is an opportunistic pathogen affecting the marine aquaculture industry (Austin and Zhang 2006). Marine vertebrates and invertebrates affected include gilthead sea bream, sea bass, common dentex, oyster, Senegalese sole, tiger prawn, groupers, flounder, puffer fish, and large yellow croaker (Tu et al. 2017; Fu et al. 2021). Virulence mechanisms of *V. harveyi* have been linked to the extracellular synthesis of hemolysins, phospholipases, proteases, and cytotoxins (Zhang and Austin 2000).

In *V. harveyi* QT520, which was isolated from diseased golden pompano-grown cages in China, three T6SS gene clusters were identified (Tu et al. 2017). The whole genome sequencing of *V. harveyi* from diseased fish collected from farms also showed three T6SS gene clusters (Fu et al. 2021). *V. harveyi* strains analyzed by comparative genomic analysis have highly conserved T6SS1 and T6SS2 genes.

Outer membrane proteins have been recognized as potential vaccine candidates. Sun et al. (2019) demonstrated that the *V. harveyi* TssJ is a putative antigen and can be used as a vaccine. The sequence analysis, western blot, and agglutination assay using the anti-TssJ antibody confirmed that the TssJ in *V. harveyi* is anchored in the outer membrane. Golden Pompano was vaccinated using two different types of vaccines: DNA vaccine (pCTssJ) and recombinant subunit vaccine (rTssJ). The use of conserved antigens in a subunit vaccine was safer than the inactivated vaccine because there is no risk of reversing the virulence (Fu et al. 2021). DNA vaccines have been proven to provide immune protection for fish, are safe to use, and are easy to produce and store (Hu and Sun 2011). After the challenge with *V. harveyi* QT520, the relative percent survival (RPS) reached 52.39% and 69.11% for rTssJ and pCTssJ, respectively. There was a significant increase in the amount of alkaline phosphatase (AKP), acid phosphatase (ACP), lysozyme, and superoxide dismutase (SOD) activity in fish vaccinated with rTssJ and pCTssJ, providing evidence that rTssJ may enhance the activation of antioxidant enzymes and lysosomes. Three to eight weeks after vaccination, both rTssJ and pCTssJ induced the production of specific serum antibodies, as determined by ELISA. Further, the transcription of immune-related genes was upregulated four weeks after vaccination and challenge in rTssJ and pCTssJ with higher folds in pCTssJ (Sun et al. 2019). These findings imply that TssJ might be a useful antigen against *V. harveyi* infections and be applied in developing vaccines.

Using the VgrG of *V. harveyi* QT520, the same group created a DNA vaccine and a subunit vaccine, which yielded higher RPS, 71.43% and 76.19% for rVgrG and pCNVgrG, respectively (Du et al. 2024). Both vaccinations successfully elicited an adaptive immunological response, resulting in increased serum antibody production against rVgrG. Fish immunized with rVgrG and pCNVgrG also exhibited increased expression of immune genes and enzyme activity.

### *2.8 Vibrio proteolyticus*

*Vibrio proteolyticus* was isolated from diseased Indo-Pacific corals and was shown to be virulent to fish and Artemia (Cervino et al. 2008; Verschuere et al. 2000; Bowden et al. 2018). The genome of *V. proteolyticus* ATCC 15338 (NBRC 13287), isolated from a marine isopod intestine (*Limnoria tripunctata*), encodes three T6SS gene clusters, T6SS modules and effectors that are not in the primary gene clusters. The

analysis of *V. proteolyticus* secretome revealed T6SS structural components such as Hcp, VgrG, PAAR-containing proteins, and putative effectors with the MIX domain, indicating the presence of functional T6SS (Ray et al. 2016). When HeLa cells were infected with *vgrG* deletion mutant (Δ*vgrG1/2/3*) in each T6SS cluster, the LDH release was the same as with the wild type, indicating that the cytotoxicity of *V. proteolyticus* is not mediated by T6SS. The T6SS3 of *V. proteolyticus* is similar to T6SS3 of *V. parahaemolyticus* (Jana et al. 2022), which was suggested to have anti-eukaryotic activity and induce inflammasome-mediated cell death in macrophages.

Similar to *V. parahaemolyticus*, the *V. proteolyticus* T6SS1 mediates antibacterial activity under warm marine-like conditions, killing activity is prominent at 30°C with 3% NaCl (Ray et al. 2017). T6SS1 delivers polymorphic effectors to both bacterial and eukaryotic targets. The antibacterial action against *E. coli* and *V. parahaemolyticus* was lost in the T6SS1 deletion mutant (Δ*vgrG1*, Δ*tssG1*), while neither deletion in T6SS2 (Δ*vgrG2*) nor T6SS3 (Δ*vgrG3*) showed any effects. The expression and secretion of VgrG1 are positively correlated with antibacterial activity. The complementation of *tssG1* rescued the antibacterial activity and secretion. Using mass spectrometry, six putative antibacterial effectors and three putative anti-eukaryotic effectors were identified in the T6SS1 gene cluster. One of the effectors encodes a MIX domain and cytotoxic necrotizing factor 1 (CNF1) domain, which are deamidases that target and activate Rho GTPases. Exogenously produced in yeast and HeLa cells cause toxicity in the latter and actin cytoskeleton rearrangements and morphological abnormalities in the former. The phenotype of catalytic site mutant suggests that the observed effects depended on a functional CNF1 domain. Moreover, no actin rearrangements were observed in RAW 264.7 cells infected with Δ*tssG1*. When *V. proteolyticus* infects eukaryotic cells, the actin rearrangements produced in macrophages but not in HeLa cells indicate that the uptake of T6SS1 by phagocytic cells may be required. The ability of *V. proteolyticus* to cause T6SS1-mediated alterations in macrophages was impaired by the deletion of the CNF1-containing effector. The complementation of this effector, but not its catalytic mutant, rescued the effect to induce actin rearrangements.

Bone marrow-derived macrophages (BMDM) undergo phagocytosis-dependent cellular death due to delivery of *V. proteolyticus* anti-eukaryotic T6SS3 effectors (Cohen et al. 2022). The activation of the NLRP3 inflammasome is caused by the effectors *tie1* (T6SS3 inflammasome-inducing effector 1) and *tie2*, which in turn cause the processing and release of caspase-1, IL-1β, and caspase-1, gasdermin D (GSDMD). The absence of GSDMD activates a compensatory T6SS-induced pathway involving gasdermin E (GSDME) and caspase-3.

In another study, T6SS3 mediated virulence in the brine shrimp *Artemia salina* nauplii (Cohen et al. 2023). The survival rate of *Artemia* nauplii was significantly lower than that of wildtype and Δ*vprh* strains when challenged with *V. proteolyticus* Δ*vprh*/Δ*hns1,* strain with an active T6SS3. VPRH is a pore-forming hemolysin. T6SS is negatively regulated by H-NS (histone-like nucleoid-structuring protein) in *Vibrio*, and deletion attenuates this effect (Salomon et al. 2014). The deletion of T6SS1 (Δ*tssG1*) and T6SS2 (Δ*vgrG2*) did not affect the Artemia survival, while the T6SS3 deletion (Δ*tssL3*) resulted in significantly higher survival of Artemia compared to Δ*vprh*/Δ*hns1*.

### 3. *Aeromonas sp.*

An important bacterial disease affecting aquaculture is Motile Aeromonas Septicemia (MAS). It is caused by motile aeromonads: *A. hydrophila*, *A. caviae*, *A. veronii* biovar *sobria* and *A. veronii* (Hanson et al. 2012; Legario et al. 2023). Disease signs include septicemia, inflammation of the anus, external and internal hemorrhages, exophthalmia, and abdominal swelling, leading to high mortality within a short period of time (Hanson et al. 2012).

#### *3.1 Aeromonas hydrophila*

As an opportunistic pathogen found in aquatic environments, *Aeromonas hydrophila* affects amphibians, birds, fishes, reptiles, and mammals. The hypervirulent *A. hydrophila* (vAh) causes outbreaks of MAS, resulting in high mortality and economic losses in cultured carp, including silver carp, bighead carp, and common carp; channel catfish, and tilapia (Ma et al. 1998; Hossain et al. 2014; Aboyadak et al. 2015).

The T6SS is encoded in seven fish pathogenic *A. hydrophila* with complete genomes (Jin et al. 2020), including large yellow croaker, diseased blunt nose sea bream, channel catfish, goldfish, and crucian carp.

Comparative genome analysis revealed that T6SS is a key genotype differentiating the vAh isolates. A virulent *A. hydrophila* GD18 isolated from diseased grass carp encodes a T6SS gene cluster with 25 conserved T6SS genes, including two VgrG. Another *hcp* and two *vgrG* are encoded outside the central T6SS cluster (Li et al. 2021a). A complete set of

T6SS gene clusters is found in vAh isolated from carp in China. In contrast, an incomplete (lack majority of the T6 core genes), encoding only *hcp1*, *tssH,* and *vgrG* was found in isolates from channel catfish in the US (Rasmussen-Ivey et al. 2016; Tekedar et al. 2019).

In particular, *A. hydrophila* NJ-35 with one complete T6SS gene cluster is encoded on a genomic island (Pang et al. 2015). *Aeromonas hydrophila* NJ-35 is associated with a high mortality rate in aquaculture. Three Hcp proteins are encoded together with a functional T6SS; one belongs to the main cluster of T6SS genes, while the other two belong to the other. In NJ-35, the three Hcp function differently in terms of environmental adaptation and virulence (Wang et al. 2018a). The secretion of *hcp* in wildtype, mutant, and complementation strains using western blot revealed that *hcp1* functions in secretion and expression of Hcp proteins. In addition, *hcp1* plays a role in T6SS-mediated antibacterial activity. Growth inhibition in *E. coli* was observed when co-cultured with single and multiple *hcp* deletion mutants (Δ*hcp1*, Δ*hcp1/2*, Δ*hcp1/3*, Δ*hcp1/2/3*) and restored after complementation of *hcp1*. On the other hand, Δ*hcp2* and Δ*hcp3* deletion mutants showed similar growth inhibition to the wild type, and *Δhcp2/3* mutant resulted in an enhanced antibacterial activity.

Compared to the wild type, the *Δhcp1* significantly decreased the adhesion to HEp-2 cells (derived from human epidermoid larynx carcinoma). On the other hand, Hcp3 positively affects bacterial adhesion, as revealed by an increase in adhesion capability in Δ*hcp2* and Δ*hcp1/2*. The ability of Δ*hcp1/2* to form biofilms might indicate that Hcp3 plays a role in biofilm formation. Whereas the ability of Δ*hcp2* to form biofilm significantly increased, indicating that Hcp2 negatively impacts biofilm formation. The virulence of all the *hcp* single, double, and triple deletion mutants except Δ*hcp1/3* were all higher than the wild-type strain suggesting that Hcp2 is involved in the virulence in zebrafish. Various *hcp* deletion mutants Δ*hcp2*, Δ*hcp1/2*, and Δ*hcp1/2/3* exhibited a significant increase in the median lethal dose (LD50).

*A. hydrophila* NJ-35 secretes type VI lipase effector (Tle1), involved in virulence and biofilm formation. Ma et al. (2020) showed that the deletion of *tle1 (Δtle1)* significantly decreased antibacterial competition ability, biofilm formation, and virulence. The Δ*tle1* had a reduced killing against *E. coli* and *V. parahaemolyticus* and an 11-fold higher LD50 in intraperitoneally injected zebrafish than the wild-type strain.

Though having an incomplete T6SS gene cluster, *A. hydrophila* ML09-119, the deletion of *hcp1* and *vgrG1* attenuated the virulence to SPF channel catfish fingerlings (Tekedar et al. 2019). ML09-119 has caused bacterial septicemia outbreaks in channel catfish cultured in the United States. After the immersion challenge, the mortality rate was significantly lower in Δ*hcp1* and Δ*vgrG1* than in wild type. Compared to control, fingerlings surviving both T6SS mutant infections and re-challenge with wildtype have survival rates of 92% and 100%, respectively, whereas control fingerlings only have survival rates of 60%.

The attenuation of virulence in *A. hydrophila* ML09-119 corroborates with *A. hydrophila* NJ-35, but the latter encodes a complete T6SS, while ML09-119 only encodes three T6SS core genes. It has been hypothesized that genomic islands associated with the Hcp and VgrG may drive functional diversification by acquiring T6SS effectors and developing evolved Hcp and VgrG proteins (De Maayer et al. 2011). Given that the T6SS in *A. hydrophila* NJ-35 is encoded on a genomic island, the mobilization of genetic islands by Hcp and VgrG may drive genomic variation in *A. hydrophila* T6SS (Tekedar et al. 2019). Future research is needed to elucidate the role of T6SS and the effect of reduced or rearranged components on the virulence of *A. hydrophila*.

Conditional regulation of T6SS was observed in *A. hydrophila*. Low temperature strongly induced the expression of *hcp*, *tle1,* and the transcriptional regulator *vasH*, while Hcp secretion was abrogated at $37^0$C. Simulating *in vivo* conditions was achieved using fish serum, assuming that T6SS expression increases during infection. As grass carp serum conditions were applied, *hcp*, *vasH*, *clpV*, and *dotU* transcriptional levels increased. Further, VasH is required for T6SS function in *A. hydrophila* strain GD18. The *hcp* transcription and expression were abolished in *vasH* deletion, indicating the inactivation of T6SS. With the deletion of *vasH* (Δ*vasH),* a significant decrease in *vgrG* and *tle1* transcription was observed. Significantly, *vasH* and *hcp* mutants abolished the antibacterial activity, suggesting that T6SS contributes to the antibacterial activity and VasH mediates the antibacterial activity in *A. hydrophila* GD18. Virulence and systemic dissemination are dependent on VasH. The LD50 value of Δ*vasH* using a grass carp infection model is $1.19 \times 10^3$ compared to $2.73 \times 10^2$ in wildtype strain. Moreover, there was a significant reduction in bacterial loads in the spleen, kidney, and liver in Δ*vasH*.

The mechanism of regulation needs to be determined in future investigations. It is probable that *A. hydrophila* strains may employ different mechanisms to adapt to different environments and hosts. Future studies of VasH could make developing therapeutics, vaccines, and antimicrobials against MAS possible.

### *3.2 Aeromonas veronii*

The spread of *A. veronii* is increasing and poses a serious threat to fish (Xu et al. 2022a, 2022b; Rahman et al. 2002). Disease outbreaks caused by *A. veronii* have been reported in cultured channel catfish in China and tilapia in Saudi Arabia (Liu et al. 2016; Hassan et al. 2017). The symptoms observed are skin ulcers, bleeding from organs, and severe ascites (Austin and Austin 2016).

The virulent *A. veronii* TH0426 isolate from Chinese yellow catfish farms possesses a full T6SS (Song et al. 2020). A component of the T6SS membrane-bound complex is the inner membrane protein DotU. The *A. veronii* TH0426 *dotU* shares 97% sequence identity with the *impK* of *Aeromonas salmonicida*. Higher expression of *dotU* in *A. veronii* TH0426 was observed compared to attenuated strain and non-virulent strain, suggesting its role in virulence regulation. Results showed that *dotU* contributed to biofilm formation and pathogenicity in *A. veronii* TH0426.

DotU deletion mutants formed 2-fold more biofilms than wild type, indicating a significant increase in biofilm formation. Moreover, the Δ*dotU* LD50 was 50-fold higher than the wild-type, suggesting that the deletion of *dotU* resulted in attenuation of pathogenicity. In addition, compared to the wild type strain, the deletion of *dotU* exhibits a lower adhesion rate to EPC cells.

Wang et al. (2023) showed that T6SS regulates motility in *A. veronii* TH0426. The *hcp* deletion mutant had reduced motility and loss of polar flagella. It has been shown that flagella influences virulence. Interestingly, the *hcp* deletion (Δ*hcp*) had an increased expression of flagella-related genes, indicating a negative feedback loop may regulate flagella-related genes in *A. veronii* TH0426. Further work is needed to clarify the mechanism of how *hcp* can regulate motility. Moreover, in comparison with the wildtype strain, the Δ*hcp* strain was significantly less capable of forming biofilms, the killing ability against *E. coli* was reduced significantly, and adhesion and invasion of EPC (endothelial progenitor cell) were lower compared with wildtype. A similar result was observed in *A. hydrophila* NJ-35. The inactivation of T6SS (Δ*hcp*) and the consequent loss of polar flagella may reduce the ability of the bacterium to adhere. The ability to form biofilm, adhere, and invade may be affected by regulating *hcp* via flagellar assembly. Furthermore, a reduction in LD50 was observed for zebrafish and crucian carp when compared with wild type strain, and a significant reduction in bacterial load was observed in crucian carp, suggesting that the T6SS or *hcp* gene can affect *A. veronii* pathogenicity by regulating flagella assembly (Wang et al. 2023).

### *3.3 Aeromonas salmonicida*

Furunculosis is caused by *Aeromonas salmonicida*, a bacterial septicemia of salmonids (Hiney and Olivier 1999). It is widely distributed and affects wild and cultured salmon, rainbow trout, Atlantic salmon, blackfin reef shark, flounder, and turbot (Cai et al. 2022). In the aquaculture industry, especially salmon farming, mortality rates can reach 100% within a week after infection.

T3SS, polymeric extracellular substances, siderophores, quorum sensing, and S-layer proteins are associated with *A. salmonicida* pathogenicity (Cai et al. 2022). Virulence factors can also be related to other secretion systems.

The *Aeromonas salmonicida* subsp. *salmonicida* A449 encodes 16 T6SS proteins on the chromosome and three T6SS genes on the plasmid (pAsa4) (Reith et al. 2008). However, it is probable that the T6SS in this strain is not functional because of several truncated T6SS genes. There is a possibility that insertion sequence elements assisted in the transfer of T6SS genes to pAsa4. Interestingly, while other species of *Aeromonas* are opportunistic pathogens, even very low levels of *A. salmonicida* infection cause disease in healthy fish (Daly et al. 1996).

*A. salmonicida* SRW-OG1 was isolated from diseased, orange-spotted grouper (Huang et al. 2020). The RNAseq results revealed that the *hcp* expression in this strain was significantly affected by temperature. Therefore, Cai et al. (2022) explored the role of Hcp in virulence by creating a stable, silent strain of *hcp*. The silencing of *hcp* decreased adhesion, growth, biofilm formation, extracellular product secretion, and enzyme activities, including protease, lipase, lecithinase, and caseinase. Importantly, groupers infected with the *hcp*-RNAi strain resulted in significantly delayed death and a lower mortality rate than the wild-type strain. The bacterial colonization in the spleen of infected

grouper in the *hcp*-RNAi group was significantly lower. Further, a high level of conservation of the *hcp* is found in *A. salmonicida* strains as well as *A. hydrophila* strains, which emphasizes its function in this group of bacteria. The prediction of the protein structure of Hcp *A. salmonicida* SRW-OG1 revealed the highest similarity with the *Vibrio cholerae* (PDBID: 5mxn1) with 78.49% amino acid sequence homology. In *A. hydrophila* NJ-35, *hcp* also affects biofilm formation, with *hcp3* and *hcp1* causing positive effects and *hcp2* causing negative effects (Wang et al. 2018a).

**4. *Edwardsiella sp.***

There are five species of *Edwardsiella*, including three species of fish pathogen (*E. piscicida*, *E. anguillarum*, and *E. ictaluri*) and two non-fish pathogen species (*E. tarda* and *E. hoshinae*) (Bujan et al. 2018). *Edwardsiella piscicida* contains pathogenic *Edwardsiella* isolates previously identified as *E. tarda* based on their phenotypic and genetic characteristics (Abayneh et al. 2013). After the reclassification, *E. tarda* includes human and environmental isolates and does not encode T6SS (Yang et al. 2012; Shao et al. 2015). Freshwater and marine fish are infected by fish pathogens, which seriously affect aquaculture worldwide.

***4.1 Edwardsiella piscicida (old name E. tarda)***

Aquaculture worldwide encounters a significant problem with Edwardsiellosis caused by *Edwardsiella piscicida*, an intracellular pathogen. In addition to its wide geographic distribution, it has been found in more than 20 fish host species, including chinook salmon, eel, channel catfish, mullet, flounder, carp, tilapia, and striped bass (Park et al. 2012; Bujan et al. 2018). The clinical signs associated with Edwardsiellosis include exophthalmia, external and internal hemorrhages, dermal ulcerations, abdominal distension, surface discoloration, and erratic swimming.

Several virulence factors have been implicated in the pathogenesis of *E. piscicida*, including the production of exoenzymes (hemolysin), presence of T3SS and T6SS, ability to adhere, invade, survive, and reproduce in epithelial and phagocytic cells (Leung et al. 2019).

*Edwardsiella piscicida* is the first *Edwardsiella* to demonstrate a critical role in the T6SS (Rao et al. 2004; Zheng and Leung 2007). The *E. tarda* virulence protein (EVP) gene cluster containing 16 genes (*evpA*-*evpO*) of strain PPD130/91 encodes T6SS (Zheng and Leung 2007). Avirulent strains were not hybridized with probes for *evpP*, *evpK*, and *evpO*, indicating a wide distribution and conservation of *evp* genes among virulent strains of *E. tarda*.

In another study, the replication rate in phagocytes, protein secretion, and virulence in blue gourami was lower in *E. tarda* PPD130/91 with deletions of *evpB* and *evpC* (Rao et al. 2004). The partial recovery of observed phenotypes was noted when *evpB* and *evpC* complementation occurred, indicating that these genes may play a role in fish phagocyte replication and could be associated with intramacrophage growth. Further functional analysis is needed to elucidate the mechanism.

The deletion of 14 T6SS genes resulted in attenuation of *E. tarda* virulence with two logs LD50 difference compared to wildtype. The *evpJ* deletion resulted in slight attenuation, but the *evpD* mutant did not differ significantly. The secretion assay demonstrated that 13 proteins, EvpA-EvpC, EvpE-EvpI, and EvpK-EvpO, are essential for the secretion of EvpC, EvpI, and EvpP. The EvpO is homologous to VasK in *V. cholerae* and IcmF1 in *P. aeruginosa,* while EvpH is a homolog of ClpV. Three proteins were secreted T6SS proteins, including EvpC (Hcp), EvpI (VgrG), and EvpP. The EvpP deletion mutant did not impact the secretion of EvpC and EvpI. However, the deletion of either *evpC* or *evpI* halted the secretion of EvpP, indicating their essential role in the secretion process. Further, EvpP and EvpC function together in the *E. tarda* cytoplasm, indicating that EvpP may target inflammasome activation in macrophages (Zheng and Leung 2007; Hu et al. 2014).

On the basis of macrophage-induced gene regulation, two putative T6SS effectors were identified, EseL and EseM (Zhang et al. 2018). EseL and EseM in the T6SS mutant (Δ*evpAB*) had a reduced translocation into HeLa cells, compared to wildtype *E. piscicida* EIB202. No immunofluorescence was detected in the HA-tagged EseL and EseM in the T6SS mutant, confirming the T6SS-dependent translocation of EseL and EseM.

EsrA-EseB, the ferric uptake regulator (Fur) protein, and the histone-like nucleoid structuring protein (H-NS) regulate the transcription of EvpP (Wang et al. 2009; Chakraborty et al. 2011; Zhang et al. 2014). EvpP expression could be downregulated by H-NS, suggesting that it could act as an *evpP* repressor. T6SS expression is regulated by Fur through EsrC (*E. tarda* secretion regulator C) in *E. tarda* strain PPD130/91 in response to changes in iron

concentration (Chakraborty et al. 2011).

EvpP can be translocated into the cytosol of infected cells (Chen et al. 2017). Also, outer membrane vesicles (OMVs) contain EvP (Park et al. 2011). It was found that EvpP was an essential effector in *E. piscicida* virulence (Xiao et al. 2008; Wang et al. 2009). In a mariculture farm in China, *E. tarda* EIB202 was isolated from diseased turbot with high mortality outbreaks due to bacterial septicemia, and the role of EvpP as a virulence determinant was investigated (Xiao et al. 2008). Zebrafish and Japanese flounder lethality was significantly attenuated by *evpP* deletion mutants, the sheep erythrocyte hemolytic activity was reduced, and *evpP* mutants failed to adhere or penetrate the Japanese flounder mucus and decreased serum resistance (Wang et al. 2009; Tan et al. 2019). The complementation of *evpP* restored the phenotypes. Importantly, in the invasion assay, the Δ*evpP* displayed deficiency in the internalization of epithelial papilloma of carp (EPC) cells, suggesting that Evp is an important component of *E. tarda* invasion mechanisms. EvpP has been implicated in several studies related to *E. piscicida* infection (Chen et al. 2017; Tan et al. 2019).

In their study, Chen et al. (2017) explored the mechanism through which the *E. tarda* T6SS interacts with eukaryotic hosts. *E. tarda* T6SS negatively regulates inflammasome activation as low NLRP3 inflammasome activity occurs during infection. The T6SS-deficient mutant, Δ*evpAB*, significantly enhanced caspase-1 activation, IL-1b secretion, and cell death in bone marrow-derived macrophages (BMDM) and J774A.1 cells (Chen et al. 2017).

T6SS-dependent transport of EvpP into HeLa cells was observed. Immunofluorescence analysis verified the injection of EvpP into host cells and EvpP positive signals were detected in HeLa cells infected with *E. tarda*. Moreover, EvpP was found to localize in the membrane fraction after *E. tarda* infection in HeLa and J774A.1 cells. In this context, EvpP can be considered a non-VgrG T6SS effector (Chen et al. 2017).

Evidently, EvpP inhibits NLRP3 inflammasome activation by suppressing ASC oligomerization that is JNK-dependent and impairs the oligomerization of ASC, an adaptor of inflammasome. In J774A.1 cells, the deletion of *evpP* (Δ*evpP*) significantly induced Jnk phosphorylation in comparison to the wildtype. A dose-dependent inhibition of Jnk phosphorylation and inflammasome activation induced by Δ*evpP* or Δ*evpAB* was observed when EvP is overexpressed in J774A.1 cells. An activated Jnk plays a critical role in NLRP3 inflammasome activation, which is dependent on ASC oligomerization. The number of ASC foci was significantly higher in J774A.1 cell infected with Δ*evpP* (Chen et al. 2017).

EvpP inhibits intracellular $Ca^{2+}$ signaling, which regulates the NLRP3 inflammasome by inhibiting intracellular $Ca^{2+}$ signaling as measured by a $Ca^{2+}$ sensitive fluorescent probe and time-lapse fluorescence reader. It was found that *evp* deletion caused J774A.1 cells to have higher intracellular calcium flux (Chen et al. 2017).

In mice, EvpP-mediated inhibition of the inflammasome promotes colonization of *E. tarda*. In the Δ*evpP*, it was observed that $Casp^{-1-1}$ mice had a significant reduction in IL-1b levels, indicating that their inflammasome activities were minimized. The Δ*evpP* elevated IL-1b levels significantly higher in wild-type and $Nlrc^{4-1-}$ mice sera compared to wild-type, suggesting an enhanced NLRP3 inflammasome activation *in vivo* (Chen et al. 2017).

These results corroborate the results of Tan et al. (2019) using an *in vivo* zebrafish larvae infection model. EvpP-mediated manipulation of Jnk-MAPK signaling *in vivo* was found to enhance Jnk activation in larvae infected with EvpP. As a result of inhibiting Jnk signaling, EvpP decreased chemokine ligand 8 (*cxcl8a*), matrix metallopeptidase 13 (*mmp13*), and interleukin-1β (IL-1β). Neutrophil-associated genes were upregulated in response to *ΔevpP* infection. When zebrafish larvae were pretreated with Jnk inhibitor SP600125, the Δ*evpP* infection-induced recruitment of neutrophils was restored. These results suggest that EvpP inhibits neutrophil recruitment.

The role of the inflammasome in regulating neutrophil recruitment was investigated using zebrafish expressing GFP driven by a neutrophil-specific myeloperoxidase (mpo) promoter (Tan et al. 2019). A caspase-1 homolog and IL-1β morpholino were injected into the zebrafish larvae embryo, then infected with selected *E. piscicida* strains. Neutrophil recruitment induced by infection with *E. piscicida* was inhibited by caspases and IL-1 in zebrafish larvae, suggesting an inflammasome-mediated mechanism. Also, higher mortality and bacterial load were observed in caspase or IL-1β zebrafish larvae. The caspase- or IL-1β morpholino knockdown larvae were more susceptible to infection and failed to restrict bacterial colonization in vivo.

A crucial component of *E. piscicida*-macrophage interactions, EvpP is required for macrophage survival and replication (Qin et al. 2020).

The *ΔevpP* had a reduced replication and survival in macrophages under oxidative and acid stress. Moreover, the *ΔevpP* caused macrophages to undergo apoptosis, as demonstrated by increased Annexin V binding and activation of caspase-3, which are involved in apoptosis. EvpP interacts with ribosomal protein S5 (RPS5) as determined by yeast two-hybrid screening and co-immunoprecipitation assays (Qin et al. 2020).

An *E. piscicida* EvpQ effector encoded by a mobile genetic element (MGE) was characterized by Li et al (2021b). Through the T6SS, EvpQ is secreted and translocated into host cells. In a blue gourami infection model, *evpQ* deletion attenuated *E. piscicida* PPD130/91 virulence. Fur negatively regulates EvpQ transcription, while EsrC positively regulates it.

### *4.2 Edwardsiella ictaluri*

*E. ictaluri* is the most important endemic infection in the catfish aquaculture industry. It was first isolated from pond-cultured channel catfish (Hawke et al. 1981) and causes enteric septicemia.

The *E. ictaluri* T6SS also consists of 16 genes, *evpA-O* (Tekedar et al. 2020). As homologs of *E. ictaluri* Eip20, Eip55, and Eip18, *E. tarda*'s EvpA, EvpB, and EvpC are expressed during an infection and are antigenic for channel catfish (Rao et al. 2004; Moore et al. 2002). The sequence analysis of Eip20, Eip55, and Eip19 showed a high identity for the ImpB, ImpC, and ImpF proteins in *Rhizobium leguminosarum* bv. *trifolii*. In *Rhizobium* and *Agrobacterium*, the T6SS is encoded in two different operons (*imp* and *hcp*). Temperature-dependent protein secretion is regulated by the *imp* operon in *Rhizobium leguminosarum* (Bladergroen et al. 2003). The expression of *E. tarda* EvpA and EvpC was temperature dependent, and the expression was suppressed at 37 $^0$C (Rao et al. 2004). Also, the survival of blue gourami is higher when challenged with bacterial cells grown at 37$^0$C, compared to cells grown at 25$^0$C.

During infection, *E ictaluri* EvpB is expressed and is highly similar to *E. tarda* EvpB (Rao et al. 2004). As a result of the deletion of *evpB*, *E. ictaluri* virulence was almost completely lost in catfish fingerlings, and *evpB* was highly internalized in catfish peritoneal macrophages (Abdelhamed et al. 2018).

Apoptosis in anterior kidney macrophages is increased by *E. ictaluri evpP* colonization in channel catfish ovary (CCO) cells (Kalindamar et al. 2020). *E. ictaluri* internalization and adhesion to catfish ovary cells were reduced when *evpP* was deleted. When oxidative stress and limited nutrients are present, *evpP* promotes survival and increases apoptosis and necrosis in catfish anterior kidney macrophages (Kalindamar et al. 2020). Compared with wildtype, the number of live macrophages in Δ*evpP* was significantly increased. Interestingly, macrophages exposed to Δ*evpP* show significantly lower necrosis than wildtype. Further, the deletion of T6SS genes Δ*evpA*, Δ*evpH*, Δ*evpM*, Δ*evpN,* and Δ*evpO* resulted in reduced replication inside peritoneal macrophages and attachment to CCO cells (Kalindamar et al. 2023). All the deletion mutants were less virulent than *E. ictaluri* wildtype in catfish fingerlings.

Other T6SS genes, *hcp1* (evpC) and *hcp2,* were also implicated in virulence through adhesion to epithelial cells and replication within catfish peritoneal macrophages, demonstrating the role of T6SS in *E ictaluri* pathogenesis (Kalindamar et al. 2021).

### *4.3 Edwardsiella anguillarum*

*E. anguillarum* ET080813T, isolated from a diseased eel containing three T6SS in Chromosome 1, has a high level of virulence in fish, but this strain has not been extensively studied (Shao et al. 2015). The T6SS2 and T6SS3 have a high level of genetic conservation along with their percent similarity, while the T6SS1 is highly conserved in *E. anguillarum*, *E. piscicida,* and *E. ictaluri*.

## 5. *Pseudomonas* sp.

*Pseudomonads* are opportunistic pathogens and are an important biological factor in the outbreak of fish disease (Rao et al. 2019). The causative agents of hemorrhagic septicemia in fish include *P. fluorescens*, *P. angulliseptica*, *P. aeruginosa*, and *P. putida* (Eissa et al. 2010). The disease is characterized by petechial hemorrhage, darkness of the skin, detached scales, abdominal ascites, and exophthalmia (Eissa et al. 2010). *Pseudomonas fluorescens* was first described as a mirror and leathern carp pathogen, and its symptoms are similar to those of motile aeromonad septicaemia.

*Pseudomonas anguilliseptica* poses a serious threat to various fish cultured in marine and brackishwater (Mekasha and Linke 2021). It causes red spot disease, a major disease of Japanese eels. *Pseudomonas aeruginosa* causes septicemia in freshwater fish, such as catfish (*Clarias gariepinus*) and tilapia (*O. niloticus*), resulting in immense economic losses in the industry (Roberts 2012). In Japan, Pseudomonas putida has only been isolated

from ayu *(Plecoglossus altivelis)* and yellowtail *(Seriola quinqueradiata)*. Unlike other pathogens in aquaculture, it has only been reported to infect tilapia and rainbow trout (*Oncorhynchus mykiss*) (Altinok et al. 2006; Salama and Gharib 2009).

To date, *P. plecoglossicida* is the only aquatic pathogen characterized by T6SS. *P. fluorescens*, *P. aeruginosa*, and *P. putida* T6SS have been characterized in plant and clinical isolates (Decoin et al. 2014; Chen et al. 2015; Bernal et al. 2017).

### *5.1 Pseudomonas plecoglossicida*

*Pseudomonas plecoglossicida* is an important fish pathogen that is causing severe economic losses in aquaculture and currently there is no efficient preventive and control measures (Mao et al., 2024). The pathogen replicates in infected fish macrophages and is a facultative intracellular pathogen (Mao et al., 2013). The first report came from ayu (*Plecoglossus altivelis*) (Nishimori et al. 2000) and subsequent reports came from twenty marine fish species, including large yellow croaker, orange-spotted grouper, yellow drum and rainbow trout (Mao et al. 2013; Huang et al. 2019; Xiang et al. 2020; Akaylı et al. 2011). Signs of disease include hemorrhagic ascites in ayu and white spots covering the liver, spleen, and kidney of affected marine fishes, leading to the term visceral white spot disease (Nishimori et al. 2000; Yuan et al., 2022).

*P. plecoglossicida*'s virulence is influenced by temperature. The bacterium induces natural infection at low (12.0–25.5°C) water temperatures (Huang et al. 2018). Aside from temperature, several studies revealed that virulence genes, such as T6SS-associated genes regulate the virulence of *P. plecoglossicida* (Tao et al. 2020; Li et al. 2022; Yang et al. 2023; Zhang et al. 2023).

The *P. plecoglossicida* strain XSDHY-P and *P. plecoglossicida* strain NB2011 encode three T6SS (T6SS-1, T6SS-2, and T6SS-3) gene clusters (Tao et al. 2018; Tao et al. 2020; Jin et al. 2021). The three clusters are all encoded in Chromosome 1 of strain XSDHY-P. Each gene cluster contains all the 13 core gene components. In *P. plecoglossicida*, T6SS1 plays a significant role in virulence and anti-eukaryotic functions. The deletion of the entire *t6ss1*, *tssH1*, and *tssD1* gene reduced *P. plecoglossicida* virulence and was unable to induce granulomas in large yellow croakers (Tao et al. 2020). Symptoms of granulation in the spleen and total mortality were observed by fish infected with WT, ΔT6SS2, or ΔT6SS3 eight days after infection. There was no evidence that the T6SS3 played a significant role in either virulence or bacterial killing.

In gram-negative bacteria, RpoE acts as a gene regulatory system to cope with stress. The knockdown of *rpoE* in *P. plecoglossicida* significantly reduces biofilm formation, swarming motility, adhesion, and virulence (Huang et al., 2019). In groupers, a mutant *rpoE* induces humoral and cell-mediated immune responses. In *P. plecoglossicida*, RpoE contributes to T6SS-mediated killing. The survival of the target *E. coli* was higher when co-cultured with *P. plecoglossicida* Δ*rpoE*. It suggests that the RIP (regulated intramembrane proteolysis) cascade is crucial for T6SS2-mediated killing activity in *P. plecoglossicida* as revealed by a significant increase in *E. coli* survival when co-cultured with deletion of RIP genes (Tao et al. 2020).

*P. plecoglossicida* gains a competitive advantage by using T6SS2 for interbacterial killing. The co-culture of *P. plecoglossicida* XSDHY-P and strains with deletion of T6SS1 (Δ*t6ss-1*) and T6SS 3 (Δ*t6ss-3*) resulted in a significant decrease in the growth of target strains *Photobacterium damselae* subsp. *damselae* 69YT1 and *E. coli* XL10 (Tao et al. 2020). Through bioinformatic analysis, four putative antibacterial T6SS effectors were identified in this strain, namely Txe1, Txe2, Txe3, and Txe4 (Li et al. 2022). Among these, three putative effectors encode DNase domain - Txe2 and Txe4 encodes a Tox-AHH toxin, while Txe3 contains an HNHc endonuclease. Txe1, Txe2, or Txe4 degraded plasmid DNA when expressed in *E. coli*, suggesting that these effectors have a nuclease activity. Interbacterial activity is mediated by only Txe1 and Txe4, with Txe1 being predominant. In Txe1, the PAAR and RhS domains are located at the N-terminus, while the C-terminus has a conserved dipeptide HH motif. Changing the catalytic site of Txe1 by site-directed mutagenesis abolished its nuclease activity and toxicity to *E. coli,* suggesting that Txe1 is indeed a nuclease effector.

Several studies have investigated the function of a single T6SS gene in the pathogenicity of *P plecoglossicida*. In *P plecoglossicida* NZBD9, Luo et al. (2019) showed no mortality in groupers infected with *clpV*-RNAi strain. The results are supported by gene expression changes of bacterial pathogens in tissue by RNA-seq and verified by virulence comparison between the *clpV* gene knockdown (*clpV*-RNAi strain) and *P. plecoglossicida* wildtype strain.

It has been proposed that VgrG, one of the structural and core components of T6SS, contributes to the pathogenicity of *P. plecoglossicida* (Yang et al. 2023). The *vgrG* deletion mutant (Δ*vgrG*) in *Acinetobacter baumannii* ATCC 19606 strain resulted in decreased

adherence to BEAS-2b human alveolar epithelial cells and impaired lethality in BALB/c mice (Wang et al. 2018b). Deleting *vgrG* (Δ*vgrG*) significantly reduced the virulence of *P. plecoglossicida*, including chemotaxis, cell adhesion, and biofilm formation (Yang et al. 2023). Transcriptome data analysis supports this finding, indicating lower expression of genes associated with adhesion, chemotaxis, biofilm, T6SS, and T3SS in Δ*vgrG* strain. *P. plecoglossicida* virulence may be affected by *vgrG* by inhibiting the secretion of virulence factors and affecting biofilm formation through quorum sensing. Moreover, the LD50 of groupers infected with the Δ*vgrG* strain was 50 times higher than wildtype strain, indicating the role of *vgrG* in *P. plecoglossicida* pathogenicity.

The deletion of *tssD* (Δ*tssD1*) is highly attenuated in large yellow croaker (Tao et al. 2020). Therefore, Ye et al. (2021) constructed a *P. plecoglossicida* strain with deletion of *tssD-1* and evaluated its efficacy as a vaccine in large yellow croaker juveniles. The live attenuated vaccine provided a significant relative percentage survival of 86.3% against wildtype *P. plecoglossicida* XSDHY-P eight weeks after primary vaccination. A significant increase in serum IgM specific to *P plecoglossicida* and immune gene expression was observed after Δ*tssD-1* vaccination. In the vaccinated fish injected with a high dose of Δ*tssD-1*, there were no clinical signs of disease, no bacteria were recovered from the spleen or kidney of surviving fish, and no mortality occurred. The *P. plecoglossicida* Δ*tssD-1* showed potential as a live fish vaccine. Further studies are necessary to assess its efficacy and understand how vaccines induce protection via cell-mediated immunity.

In a recent study, Zhang et al. (2023) showed that the RpoE of *P. plecoglossicida* positively controls T6SS expression in a temperature-dependent manner. RIP signal cascades activate RpoE to regulate the expression of genes in response to environmental stimuli. RNA sequencing showed that *rpoE* was significantly expressed at virulent temperature (18°C) compared to non-virulent temperature (28°C).

Transcriptome sequencing of *P. plecoglossicida* incubated at virulent (18$^0$C) and non-virulent (28$^0$C) temperatures showed that *rpoE* was significantly expressed at 18$^0$C (Huang et al. 2018). A challenge test in orange-spotted grouper showed that *P plecoglossicida*'s virulence is significantly reduced when *rpoE* is knocked down, suggesting that *rpoE* contributes to its pathogenesis (Huang et al. 2019). The RNA sequencing results revealed that at 18$^0$C, the transcription of T6SS2 genes was downregulated in the *rpoE* deletion mutant.

According to qRT-PCR analysis, T6SS2 gene transcript levels were significantly downregulated in *the rpoE mutant compared to the wild type* at 18$^0$C. RpoE deletion does not induce *hcp2* transcription, which indicates a temperature-dependent regulation of T6SS2 by RpoE. When host cells are exposed to low temperatures, the secretion of T6SS1 is strongly induced.

Interestingly, the expression of *hcp1* was not affected by temperature regulation. However, *rpoE* deletion affects *hcp1* expression and secretion. Thus, it may be possible that RpoE is involved in T6SS1 and T6SS2 expression, with T6SS2 exerting a dominant control.

The possible role of RpoE for infection in eukaryotic cells has been investigated. The replication of the T6SS-2 deficient *P. plecoglossicida* strain exhibited a reduction in macrophage J774A.1 compared with wildtype (Jin et al. 2021). *Pseudomonas plecoglossicida*-infected cells exhibited severe cytoplasmic vacuolation and dying morphology, while Δ*rpoE*-infected cells had fewer lesions. Moreover, there was a lower level of intracellular replication of ΔT6SS2 in J774A1 than in wildtype. These results indicate RpoE regulates virulence determinants such as T6SS2 and other pathways associated with intracellular replication of *P. plecoglossicida* in macrophages.

### *6. Francisella noatunensis* subsp. *orientalis* (Fno)

Fish mortality rates range from 30-75% in marine and freshwater environments due to *Francisella noatunensis*, an emerging pathogen affecting both wild and farmed species (Hsieh et al., 2006; Olsen et al., 2006; Birkbeck et al., 2007). Two genetic types of *Francisella* spp. cause disease in cold and warm water fish species, respectively: *Francisella noatunensis* subsp *noatunensis* (Fnn) and *Francisella noatunensis* subsp *orientalis* (Fno) (Lewis and Soto, 2019; Mikalsen et al., 2009; Kamaishi et al., 2005). Colonization and replication of *F. noatunensis* occur in phagocytes and endothelial cells (Soto et al., 2017). As a result of the disease, multiple organs, especially the kidney and spleen, experience severe granulomatous inflammation (Birkbeck et al., 2011). Higher mortalities are observed at temperatures below 28 °C (Colquhoun and Duodu, 2011).

The Fno genome contains several virulence determinants, including gene sequences that might encode proteins similar to *F. tularensis* T6SS components, including IglA (TssA homolog), IglB (TssB homolog), VgrG, DotU, and PdpB (Clemens et al. 2018). The gene transcription analysis of T6SS

homologs in Fno, suggests its role in pathogenicity (Lewis and Soto 2019). Higher expression of T6SS homologs was observed at lower temperatures (< 30°C) and when exposed to $H_2O_2$, suggesting a role in oxidative stress tolerance. A better understanding of the pathogenicity mechanism employed by Fno requires further research.

The genome of *Francisella* encodes a Francisella Pathogenicity Island (FPI), which represents a unique T6SS subtype, T6SS$^{ii}$ (Nano and Schmerk 2007; Russell et al. 2014). PdpA encodes the pathogenicity determinant protein A in FPI, which is conserved in *Francisella* and used to secrete effectors by the *Francisella* T6SS (Schmerk et al. 2009; Eshraghi et al. 2016; Hansen et al. 2021).

Using a specific-pathogen-free zebrafish infection challenge through intraperitoneal injection, Hansen et al. (2021) demonstrated that *pdpA* deletions in Fno resulted in attenuated virulence, impaired intracellular replication and cytotoxicity, and the complementation restored wildtype levels of virulence. No mortality and histopathological changes were observed. Wildtype challenge test revealed significant protection from an acute lethal dose after immunization with Fno Δ*pdpA*. A study in Nile tilapia by de Alexandre Sebastião et al. (2022) evaluated the efficacy of a mutant *pdpA* as a live attenuated vaccination. Immunized tilapia had a 45% relative percent survival, and no clinical signs associated with Francisellosis were observed after the immersion challenge. Comparing the vaccinated and non-vaccinated fish, the vaccinated fish had significantly higher IgM levels. These studies revealed the potential use of attenuated Fno stains as a vaccine against Francisellosis in farmed and wild fish.

### *7. Flavobacterium sp.*

Worldwide, *Flavobacterium* is an important pathogen of wild and cultured fish (Wahli and Madsen 2018). In salmon and channel catfish farming, *F. psychrophilum*, *F. branchiophilum,* and *F. columnare* have been reported to cause economic losses. Bacterial coldwater disease in freshwater fish, bacterial gill disease, and columnaris disease are caused by *F. psychrophilum*, *F. branchiophilum,* and *F. columnare* (Wahli and Madsen 2018). Other *Flavobacterium* species, including *F. johnsoniae*, *F. succinicans*, *F. hydatis*, *F. chilense*, *F. araucananum*, *F. spartansi*, *F. plurextorum,* and *F. tructae* have been associated with diseased fish (Zamora et al. 2012; Loch and Faisal 2014a; Loch and Faisal 2014b; Wahli and Madsen 2018). Despite extensive research, no effective methods have been proposed to reduce its devastating effect.

To date, only comparative gene analysis has been done in *Flavobacterium* fish pathogens. *F. branchiophilum*, *F. araucananum*, *F. chilense*, *F. spartansii*, and *F. tructae* encode a complete T6SS, while *F. columnare*, *F. hydatis*, and *F. plurextorum* encode partial T6SS (Tekedar et al. 2017; Kumru et al. 2020). Interestingly, multiple *tssD* is encoded by *F. columnare*, *F. branchiophilum,* and *F. johnsoniae* (Tekedar et al. 2017). Considering *Flavobacterium*'s high prevalence of T6SS, further research is warranted to determine how it impacts pathogenicity and environment adaptation.

### 8. Conclusion/Future Perspective

By better understanding T6SS in aquatic pathogens, we can develop diagnostic tools, more effective therapeutics, and vaccines for mitigating diseases in the aquaculture industry. Interestingly, comparative genome analysis revealed that virulent *A. hydrophila* and AHPND-causing *V. parahaemolyticus* may be distinguished from other strains by the presence of T6SS (Pang et al. 2015; Li et al. 2017). Following the genome studies, the next step in future studies will be to characterize the functional properties of T6SS in *V. crassostrea*, *E. anguillarum*, and *Flavobacterium* spp. Most of the T6SS functions in aquatic pathogens described above are antibacterial activity, pathogenicity, regulation of motility (Huang et al. 2019; Wu et al. 2023), and modulation of microbiome composition (Tang et al. 2022) (Table 2). Not many antibacterial and virulence effectors have been characterized (Figure 1).

T6SS demonstrates antibacterial action against a variety of bacteria, primarily gram-negative bacteria and, in the case of *V. parahaemolyticus*, gram-positive bacteria (Wang et al. 2022). *In vivo* competition assay demonstrated a reduced killing by the T6SS deletion mutant against target competitors in oyster and zebrafish hosts (Hubert and Michell 2020; Ma et al. 2020). Specific T6SS effectors, such as lipase and nuclease, were implicated in the killing activity in *A. hydrophila* and *P. plecoglossicida*, respectively (Ma et al. 2020; Li et al. 2022).

Several *in vitro* studies demonstrated how T6SS plays a role in the colonization of host cells and the cytotoxic activity in various cells/cell lines through exogenous expression and deletion mutants. For instance, reduced translocation of *E. piscicida* effectors was observed in T6SS mutant (Δ*evpAB*) in HeLa cells (Zhang et al. 2018) whereas the exogenous expression

of *V. proteolyticus* effector induced cytoskeleton rearrangements, morphological abnormalities in yeast, and toxicity in HeLa cells.

T6SS has been shown to mediate virulence *in vivo* using brine shrimp nauplii, catfish fingerlings, grass carp, crucian carp, orange-spotted grouper, Japanese flounder, and blue gourami (Table 2). The deletion or inactivation of the T6SS gene resulted in attenuation of pathogenicity. Studies demonstrating the histopathology of wild-type vs. T6SS mutant may support the possibility that T6SS is directly involved in causing the disease. Identifying the effectors directly involved in pathogenesis will also be of interest.

Temperature, salinity, and quorum sensing mediate the regulation of T6SS. However, the conditions, cues, and mechanisms that regulate T6SS activity for many of the T6SS studied to date are still unknown.

Bacterial secretion systems are promising targets for developing anti-virulence drugs, but when inactivated, they can result in pathogen attenuation or loss of virulence (Baron and Coombes 2007). Developing anti-virulence drugs targeting conserved T6SS components, such as extracellular apparatus components, may be possible. T6SS components in *V. harveyi* and *P. plecoglossicida*, described above, have been employed as antigens in the production of vaccines (Sun et al. 2019; Ye et al. 2021). Yang et al. (2015) developed a live attenuated vaccine (LAV) for turbot aquaculture using *Edwardsiella tarda* YWZ47 *esrB* mutant with low T6SS and T3SS secretion. The LAV was administered to turbot (*Scophthalmus maximus*) through immersion and subsequently challenged with the wildtype *E. tarda* strain EIB202. The YWZ47 could confer a high protection rate and higher RPS (64.4 %) for vaccinated fish compared to Δ*esrB,* with only 51.1% RPS. The vaccine can be administered via immersion, making it ideal for use in aquaculture. An attenuated *E. ictaluri* vaccine was tested by Abdulhamed et al. (2018) using a strain with an *evpB* mutation. The vaccine provided protection to catfish fry and fingerlings challenged with *E. ictaluri* wildtype with 80.34% survival. Another study (Kalindamar et al. 2023) showed the efficacy of vaccination using T6SS mutants in channel catfish fingerlings after a challenge with *E. ictaluri*. All the T6SS mutants provided better protection than the control, especially Δ*evpD*, Δ*evpE*, Δ*evpG*, Δ*evpJ*, and Δ*evpK*. While Δ*evpA*, Δ*evpH*, Δ*evpM*, and Δ*evpN* caused less protection.

Several studies have demonstrated that secretion systems can deliver recombinant proteins (Ittig et al. 2015; Simon et al. 2015; Walker et al. 2017; Bai et al. 2018). *Yersinia enterocolitica* T3SS substrate YopE allows fast and controlled delivery of bacterial, viral, and human proteins to target cells using the injectisome of extracellular bacteria (Ittig et al. 2015). Through nanobody-fusion proteins, multiple proteins can be simultaneously injected and targeted to different subcellular locations. Proteins can be released from the YopE fragment by T3S-translocated viral proteases or fusion with ubiquitin and cleavage by endogenous ubiquitin proteases after delivery. T3 Pharmaceuticals is currently doing a clinical trial using the system to deliver therapeutic proteins to target cancer cells selectively. Promising results showed immune cell activation and regression of primary tumor and metastasis.

Ting et al. (2020) generated strains expressing surface-displayed antibodies or nanobodies directed at the target cell's unique surface antigen. The system makes use of the antibacterial activity of the T6SS to eradicate specific bacteria from the polymicrobial community. A nanobody recognizing an antigen on the cell surface of aquatic bacterium or virus can be generated to control important diseases in aquaculture. This system can be integrated with the platform developed by Jana et al. (2021) for controlled delivery of effectors to improve the specificity of biocontrol strains to target aquatic bacterial pathogens. Jana et al. (2021) introduced an exogenous antibacterial T6SS from *Vibrio parahemolyticus* into *Vibrio natriegens* and engineered an on/off switch to activate T6SS in response to an external cue.

Another recent development in the field is engineering the T6SS to deliver an exogenous effector and Cre recombinase, a genetic editing protein, into target bacteria (Hersch et al. 2021). The system can manipulate the microbiome or act as a next-generation antimicrobial.

One of the most prevalent diseases affecting aquaculture is fungal infection. T6SS effectors targeting fungal cells have been reported. The delivery of T6SS antifungal effectors in *Serratia marcescens* leads to fungal cell death (Trunk et al. 2018). Another study described an effector in *Klebsiella pneumoniae* that can target bacteria and yeast (Storey et al. 2020). Effectors with activity against aquatic fungal pathogens will likely be identified in the future. This is another direction to harness the T6SS in aquatic animal health management.

The use of T6SS-encoding bacteria as probiotics is possible through symbiotic bacterial strains or engineered strains. We have seen some

potential of this T6SS application in controlling plant pathogens (Bernal et al. 2017; Decoin et al. 2014). In addition, T6SS activity has been shown to be affected by host and dietary components. The study by Bachmann et al. (2015) demonstrated that microbiota modify bile acids to inhibit the production of T6SS in pandemic *V cholerae*. A future application might be to modulate microbiota behavior using specific dietary components to control pathogenicity. Lastly, this can lead to the development of vaccines targeting secreted virulence factors as prophylactic strategies.

## CONFLICTS OF INTEREST

The work was carried out without any financial or commercial ties that might be seen as having a conflict of interest.

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